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SCREENING OF ANTIBIOTIC (STAUROSPORINE) PRODUCING ACTINOBACTERIA (*Streptomyces sp.*) FROM TERRESTRIAL ENVIRONMENT SOIL OF THANJAVUR DISTRICT, TAMILNADU, INDIA

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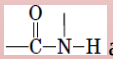
ABSTRACT
Actinobacteria have gained prominence in recent years because of their potential for producing antibiotics which are not only used in pharmaceutical industries but also in the agriculture practice to inhibit the growth of several phyto-pathogens. A total of 5 different types of actinobacteria were isolated from the soil of terrestrial environment from Thanjavur District. All the isolated actinobacteria were characterized and identified based on the morphological, biochemical and molecular characteristics. The isolates were belonged to the genera *Streptomyces* KV1, KV2, KV3, KV4 and KV5. The isolated strains were screened by cross streak and well diffusion methods. Only *Streptomyces* sp KV2 was selected for further studies. The result of the molecular characteristics selected *Streptomyces* sp. (KV2) identified as *S. champavatii* (KV2). The result of the screening revealed that the crude extract was effective against human and plant pathogens. The antimicrobial efficacies and solubility of the antimicrobial compound was evaluated and extracted with organic solvents. Extracted antimicrobial compound of the *Streptomyces* sp. KV2 was characterized by using UV, FT-IR and ^{1}H and ^{13}C NMR spectral analyses. Rf value in the TLC chromatogram characterized that, the metabolite comes under  , -O-H, -C-O-CH_3, F-CH_3,  and  group very much related to Staurosporine.

KEY WORDS: Terrestrial soil; *Streptomyces* sp. KV2; antimicrobial activity; UV; FT-IR; NMR analyses.

INTRODUCTION
Actinobacteria are filamentous, sporulating bacteria with high G+C (57-75%) content in their DNA. They are free living, saprophytes and a major source for the production of antibiotics (Arifuzzaman *et al*., 2010) confirmed to be a promising antagonistic bacterium against several pathogens and are well known for their potentiality to produce a large number of biologically active metabolites used in industry and pharmacy (Baltz, 1998). Especially, *Streptomyces* are prolific and can produce 1/3 of antibiotics and active secondary metabolites commercially available now.

The majority of actinobacteria are free living, saprophytic bacteria widely distributed in soil, water and colonizing plants. The actinobacteria are noteworthy as antibiotic producers, making three quarters of all known products and other class of biologically active secondary metabolites (Okami and Hotta 1988).

Terrestrial actinobacteria have been of great global interest to scientists for the past fifty five years, pertaining to the discovery of novel genera with newer bioactive metabolites. During the recent decades, the marine actinobacteria are recognized as a source of novel antibiotics, anticancer agents and other novel secondary metabolites with unusual structure and properties. But, actinobacteria have been primarily isolated from the terrestrial sources only, and the first report of actinobacteria being recovered from marine sediments appeared few decades ago. It is suggested that, the terrestrial actinobacteria are run-off into the sea by river water/flood where they would stay viable for years as terrestrial contaminating microorganisms (Goodfellow and Haynes, 1984). Even though, the rate of discovery of new compounds from terrestrial actinobacteria is decreasing (Okami and Hotta, 1988), it is crucial that new groups of terrestrial actinobacteria from unexplored or underexploited habitats be pursued as sources of novel bioactive secondary metabolites.

The terrestrial actinobacteria have potential biotechnological applications, and are an original resource for structurally diverse secondary metabolites (Okami and Hotta, 1988). *Streptomyces*, a representative genus of actinobacteria that is mainly of terrestrial soil origin, has accounted for the production of 80% of antibiotics which are useful also in agricultural industries (Thakur *et al.*, 2007; Singh *et al.*, 2006). The wide distribution of *Streptomyces* in soil and their ability to produce novel antibiotics and non-antibiotic lead molecules had caused these bacteria to be targeted in drug screening programme. Recently, many studies have been carried out on the isolation, characterization and genotyping of soil *streptomycetes*. Unfortunately, studies on discovery/re-discovery of novel antibiotics is essential to control the drug/multi-drug resistant pathogenic microorganisms are less or unsuccessful. Therefore, the present study has undertaken to demonstrate the antimicrobial potential of *streptomycetes* isolated from different habitats in terrestrial soils of Thanjavur district, Tamilnadu, India.

MATERIALS AND METHODS

Sample collection: The soil samples were collected from rhizosphere regions (Thanjavur District, Tamiladu) at a depth of 15cm in December 2010. The samples were transferred to new polythene bags using sterile spatula. The samples were transported to the laboratory for the isolation of actinobacteria.

Isolation of actinobacteria (Porter *et al.*, 1960): Sample was air dried aseptically. After a week sample was incubated at 55ºC for 5 minutes in order to facilitate the isolation of actinobacteria. Then tenfold serial dilution was prepared with one gram of soil sample using distilled water. Samples were inoculated on Starch Caesin agar. Streptomycin (30µg/l) and nystatin (50µg/l) were added to the medium in order to retard the growth of bacteria and fungi respectively. All the plates were incubated at 28± 2ºC for 7 – 10 days. Colonies with suspected actinobacteria morphology (i.e., isolates with white and brown colonies) were purified. The pure cultures of the actinobacteria were streaked on SCA medium plates.

Preliminary characterization of actinobacterial isolates

Colony morphology (Burkholder *et al.*, 1954): Colony morphology of the purified actinobacterial isolates on SCA medium were recorded with respect to colour of aerial spore mass, size and nature of the colonies, colour on the reverse side and diffusible pigmentation.

Light microscopy (Coverslip culture technique - Kawato and Shinobu, 1979): Purified actinobacterial culture plates were prepared and 3 to 4 sterile coverslips were inserted at an angle of 45°. The plates were incubated at 28±2°C for 4-8 days. The coverslips were removed at 2-3 days of interval and observed under the high power magnification. The structure and arrangement of conidiospores and arthospores on aerial and substrate mycelia were observed and compared with Bergey's Manual of Determinative Bacteriology.

Screening of antimicrobial compound producing actinobacteria

Test bacteria and fungi: Three different Gram positive bacteria (*B. subtilis, S. aureus* and *S. pneumoniae*), three Gram negative bacteria (*E. coli, S. typhi* and *K. pneumoniae*), and four fungal pathogens namely *Fusarium* sp., *A. niger*, *A. flavus* and *A. fumigatus* were employed for screening of antibiotic producing ability by the actinobacterial isolates. All the test microorganisms were obtained from Microbiology Laboratory, Bharathidasan University Constituent College for Women, Orathanadu - 614 625, India, they were compared with Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained for further purpose.

Preliminary screening: Single streak of the selected actinobacterial strains was made on the surface of the nutrient agar medium, and incubated at 28±2°C. After observing a good ribbon like growth of the actinobacteria at the center of the plates, the pathogens were streaked at right and left angles to the original streak of actinobacteria and incubated at 37^{o}C for 24 h. Based on the presence and absence of inhibition zone, the antimicrobial compound producing actinobacteria were selected. Further, the isolates producing zone of inhibition of 4 mm and above was only considered as antibiotic producers and selected for secondary screening.

Secondary screening: The selected actinobacteria with antimicrobial activity in preliminary screening were further subjected to secondary screening by well diffusion assay. All the active actinobacterial isolates selected in primary screening were inoculated into the flasks containing 50 ml SCB and incubated in an orbital shaker (at 200 rpm) at 30°C for 6 days. After incubation, the broth cultures were centrifuged at 10,000 rpm for 10 min. Supernatant was collected aseptically and their antimicrobial properties were tested against test bacteria and fungi.

Extraction of crude antibiotic metabolite (Liu *et al.*, 1986): Starch casein broth was inoculated with *Streptomyces* sp. (KV2) culture and incubated in shaker at 28˚C/120 rpm for 5-7 days. Fermented broth was filtered and subjected for solvent extraction method to recover the crude antibiotic metabolite in pure form. Organic solvents namely (Acetone, chloroform, ethanol, ethyl acetate, methanol, petroleum ether and xylene) were added to the filtrate in the ratio of 1:1 (v/v). The mixture was shaken vigorously for 30 min complete extraction. Organic solvents phase contains antibiotic substances, separated from aqueous phase. It was evaporated to dryness in water bath at 80°C-90°C and

the residue obtained was used to determine antimicrobial activity.

Characterization of *Streptomyces* sp. (KV2): Selected actinobacterial isolate *Streptomyces* sp. (KV2) was identified using standard International Streptomyces Project (ISP) procedure. Morphological identification of isolated colonies was carried out by phase contrast microscope, scanning electron microscope, simple staining, Grams staining and motility testing by hanging drop method.

Biochemical characterization (Shirling and Gottlieb, 1966): Various biochemical characterizations were characterized by using standard methods.

Molecular characterization of streptomycetes sp. (KV2): Streptomycetes were grown upto the late exponential phase in starch casein broth at 28±2°C, cells were harvested and washed twice with Tris EDTA buffer or 10.3% sucrose prior to DNA preparation. PCR amplification, secondary structure prediction, evolutionary tree analyses were performed standard methods. In order to identify the isolates by molecular methods, PCR and 16S rRNA sequencing were carried out.

Separation and purification of antimicrobial compounds: The concentrated ethyl acetate extract was subjected to primary analysis of the antibacterial substances. It was performed by thin layer chromatography (TLC) on silica gel slides by using n-butanol-ethyl acetate-water (9:9:1) as a solvent system. Chromatograms were observed under UV light and exposed to iodine vapours.

Characterization of antimicrobial compounds (Harindran *et al.*, 1999).

Qualitative analysis of functional group of the antimicrobial compounds

Functional group analysis was performed in order to find out the classes or nature of antimicrobial compounds group.

Quantitative analysis of functional groups of the antimicrobial compounds (Ivanova and Schlegel, 1997).

GC-MS analysis

The selected streptomycetes sp. (KV2) with potential antimicrobial activity were inoculated into 100 ml of SCB and incubated at 30°C in a shaker at 200 rpm for 6 days. The broth culture was filtered using Whatman No. 1 filter paper (11 µ) followed by Millipore filters (0.45 µ). Organic solvents namely ethyl acetate were added to the filtrate in the ratio of 1:1 (v/v). The mixture was shaken vigorously for 5 min. supernatant was collected and preserved at 4°C. The GC-MS spectrum was recorded by using GC-MS QP2010plus (Column: RTX5MS, detector: Mass spectrometry).

Ultra violet spectrum

The UV spectral measurement of the pure compound obtained from fermentation broth of *Streptomyces* sp. KV2 was made 200-400 nm by using Shimitzu (UV 1601) instrument; ethanol was used as a solvent.

Fourier Transfer-Infra Red spectrum

The pure compound of *Streptomyces* sp. KV2 was subjected to IR spectral analysis. IR spectrum was recorded on a Bruker FT-IR instrument equipped with AT-XT Golden gate accessories.

NMR spectral analysis

The pure antimicrobial compounds were characterized by ^{1}H and ^{13}C NMR spectroscopic experiments recorded on an Avance 400 MHz NMR spectrophotometer with $CDCl_3$ as solvent and TMS as internal standard.

RESULT

Totally, 40 actinobacterial colonies were isolated from various terrestrial soil samples of (Kanniyakurichi village) Thanjavur district, Tamilnadu. From these 40 colonies of actinobacteria, morphologically varying colonies were purified, sub-cultured and stored at 4°C on SCA medium for further studies (Plate I). Among the 40 actinobacterial colonies, only 5 isolates were morphologically different.

From these 40 colonies of actinobacteria, morphologically variying colonies were purified, sub-cultured and stored at 4°C on SCA medium for further studies (Plate II). Colours of aerial spore mass of the isolates were categorized into 2 groups including white and brown series on SCA medium. Among these 2 groups, most of the isolates were produced white colour series than brown series.

Screening of antimicrobial compound producing actinobacteria

Preliminary screening

In the primary screening of antimicrobial activity, out of 5 isolates, 4 (80%) isolates exhibited antimicrobial activity (Table 1). Among 4 antimicrobial activity possessed isolates, all the 4 isolates had antimicrobial activity. Among 4 antibacterial isolates, 3 isolates had activity against Gram positive bacteria, 4 against Gram negative bacteria. Individually, 4 isolates possessed activity *A. fumigatus* and 3 isolates activity against *Fusarium sp.*, *A. niger* and *A. flavus* activity.

Secondary screening

In the secondary screening, 3 out of 4 actinobacteria with notable antimicrobial activities were further evaluated for their activity against test bacterial and fungal pathogens. Out of 3 antagonistic actinobacteria, the actinobacterial isolate, *Streptomyces* sp. (KV2) was found strong antagonistic activity against all the 6 bacterial and 4 fungal pathogens tested than other actinobacterial isolates (Table 1). Hence, these isolates

Streptomyces sp. (KV2) was justifiably selected for further investigations.

Antimicrobial efficacies of selected streptomycetes

The antimicrobial efficacies of the two isolates *Streptomyces* sp. (KV2) were evaluated with seven solvent extracts against the bacterial and fungal test organisms. The isolates possessed maximum antimicrobial activities when it was extracted with ethyl acetate and petroleum ether. The isolate namely *Streptomyces* sp. (KV2) had maximum antimicrobial activity in ethyl acetate against *E. coli* (14 mm), followed by *A. fumigatus* (13 mm), *S. pneumoniae* (12 mm), *Fusarium* sp. (10 mm), *A. niger* (9.5 mm), *B. subtilis* and *A. flavus* (9 mm), *S. typhi* (8.5 mm) and *S. aureus* (6 mm), no activity was found against *K. pneumoniae* (Table 2).

Characterization and identification of selected *Streptomyces* sp. (KV2)

The two isolates *Streptomyces* sp. (KV2) found to have broad spectrum antimicrobial activities were selected for further characterization on the basis of morphological, biochemical and molecular characteristics (16S rRNA gene sequencing).

Microscopic observation of *Streptomyces* sp. (KV2)

Based on the light and scanning electron microscopy, the antimicrobial producers *Streptomyces* sp. (KV2) formed straight to flexuous (rectiflexibiles) spore chain on aerial mycelium with smooth surface. The microscopical studies of the isolates undoubtedly placed these isolates under *Streptomyces* genera (Plate III).

Biochemical characterization

Among the various parameters studied, positive result were observed with potential isolates *Streptomyces* sp. (KV2) in production of catalase, urease and H_2S hydrolyses of casein, starch, esculin and lecithin, and negative results were observed in production of β-lactamase, melanin, DNase, RNase and xanthine, nitrate reduction test, hydrolyses of gelatin and lipid and heamolysis test.

Cultural characterization

Cultural characteristics of the two isolates were studied with seven different culture media. Both the isolates *Streptomyces* sp. (KV2) produced grey, white and ash coloured spore mass and white, brown and yellowish reverse side in most of the media tested. None of the isolate produced diffusible pigments on any of the nine media tested (Table 3).

Molecular characterization of *Streptomyces* spp.

The molecular characteristics of *Streptomyces* sp. (KV2) were carried out by PCR amplification of 16S rDNA gene and their sequencing. The sequences of both *Streptomyces* sp. (KV2)16S rRNA genes were deposited in genbank http://www.ncbi.nlm.nih.gov/genbank and received the accession numbers KF454869. The sequences of the two isolates were compared with sequences of already existing species of streptomycetes from EMBL database to determine the phylogenetic relatedness using Neighbor joining tree method. It was revealed that, about 361 bp sequence of the isolate *Streptomyces* sp. (KV2) found 98% similarity with the existing species of *S. champavatii* (iafA).

The secondary structure of 16S rRNA gene of *Streptomyces* sp. (KV2) showed 22 stems in their structure. However, isolate are in energy thresh hold, cluster factor, conserved factor, compensated factor, conservativity, part of sequence, greedy parameters and treated sequence as indicated by RNA fold web server software. The restriction sites found in both *Streptomyces* isolates are shown. Totally, 52 restriction enzyme sites were observed in *Streptomyces* sp. (KV2. The GC contents of *Streptomyces* sp. (KV2) were found to be 60%.

Identification of potential *Streptomyces* sp.

Based on the morphological, biochemical, and quite significantly molecular features, antimicrobial compound producing *Streptomyces* sp (KV2) was identified as *S. champavatii* (KV2).

Extraction of antimicrobial compounds: The fermented broth containing antimicrobial compounds of selected potential streptomycetes was extracted with eight different solvents. The compound of *S. champavatii* (KV2) dissolved completely in ethyl acetate followed by DMSO and weakly in methanol and chloroform, whereas it does not dissolved in acetone, xylene, petroleum ether and water. The extracted compounds were assessed for their antimicrobial ability (Table 4).

Separation and purification of antimicrobial compounds: The ethyl acetate extracted compounds of the two isolates were purified and separated by thin layer chromatography. Single separated bands were observed and the Rf value was 0.49 cm for *S. champavatii* (KV2). The Rf value of the separated compound was calculated by using the following standard formula.

$$\text{Rf values} = \frac{\text{Distance traveled by the solute (Compound)}}{\text{Distance traveled by the solvent}}$$

S. champavatii (KV2) compound– Spot 1: Rf value = 3.7/7.5 = 0.49

Characterization of antimicrobial compounds

The separated *S. champavatii* (KV2) compound was pale yellow in colour, viscous nature and melting point was 145°C. The compound was stable at pH from 4 to 7 and the temperature ranging from 30 to 45°C. It showed positive reactions to silver mirror test for aldehyde and ketone, ninhydrin test for protein and peptide, Elson-Morgon test for amino acid. It showed negative reaction to Benedict's test (absence of sugar moiety), Molish's

test (absence of reducing sugar) and Bial's test (absence of pentose sugar).

GC-MS analysis of the ethyl acetate solvent extract was performed and the compounds were identified based on their retention time. The following chemical compounds were reported from *Streptomyces* sp. (KV2) such as acetic acid, 1-methylpropyl ester (CAS) 2-butyl acetate (3.250 min), butyl acetate (3.350 min), acetic acid, 2-methylpropyl ester (CAS) isobutyl acetate (3.450 min), benzene, ethyl- (CAS) EB – ethylbenzene (5.00 min), methyllaurate (5.100 min), benzene, 1,2-dimethyl- (CAS) o-xylene (5.00), benzene, 1,4-dimethyl- (CAS) p-xylene (5.140 min), benzene, 1,3-dimethyl- (CAS) m-xylene (5.14 min), pyrimidine, 2-methoxy-5-methyl (18.698 min), pyridine, 2-methoxy-5-nitro- (22.057 min), pyrimidine-2(1H)- thione, 4,4,6-trimethyl-1-(1-phenylethyl)- (29.023 min) and l-phenylalanine, N-(2,6-difluorobenzoyl)-, methyl ester (29.291 min) (Figure 1). Benzenepropanoic acid (16.391min), sulfurous acid, 2-ethylhexyl hexyl ester (17.063 min), 2- hexenoic acid, 5-hydroxy-3,4,4-trimethyl-, (E)- (21.408 min), 1,2-benzenedicarboxylic acid, butyl 2-methylpropyl ester (23.468 min), n-hexadecanoic acid (23.468 min), phosphonic acid, bis(1-methylethyl) ester (24.028 min), octadecanoic acid (25.327 min) and 1-phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodeca hydro-1,4a,7-trimethyl-, methyl ester, [1R-(1-alpha,4a (27.813 min), pyrrolo[1,2-a] pyrazine-1,4- dione, hexahydro- (21.587 min) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (26.940 min) and p-benzoquinone, 2-hydroxy-5-(methylthio)- (25.932 min), propenylguaethol (17.175 min), phenol, 3-methoxy- (17.555 min) and phenol, 3, 5-dimethoxy- (22.416 min), tridecane, 1-iodo- (20.669 min), tridecane, 5-propyl- (20.736 min), 7-Acetyl-1,7-diazabicyclo[2.2.0] heptane (21.206 min), heneicosane (21.744 min), nonadecane (22.774 min), 1,4-dioxaspiro[4.5] decane, 6-methylene- (23.222 min), heptadecane (24.678 min), hexacosane (28.821 min), triacontane (29.560 min), nonacosane (31.240 min), tetracosane (33.592 min), caprolactam (15.248 min) and squalene (30.658 min) (Figure 1).

The antimicrobial compound of *Streptomyces* sp. KV2 revealed that the absorption maximum was 204, 212, 218 and 226 nm in ethyl acetate. The UV spectrum of *Streptomyces* sp. KV2 compound was showed in figure 2. FT-IR spectrum of the compound showed two absorption peaks in the regions of 3440, 2926 and 2857 cm^{-1}. The spectrum of the antimicrobial compound indicated the presence of alcoholic, phenolic and alkenes groups. The absence of Br stretch alkyl halides (C-Br), aliphatic amines, carboxylic acid (COOH), ester (COOR) and alkynes (C=C), was confirmed by the lack of bands in the region of 690-515, 1250-1020, 1250-1020, 1700-1750 and 2000-2060 cm^{-1} respectively (Figure 3).

Large numbers of peaks throughout the δ (chemical shift) value of 0-10 were observed in the ^{1}H NMR spectrum of purified *S. champavatii* (KV2) compound. The peaks (chemical shift) values 1-4, 2-5, 3.3, 4.2, 6-8 and 7-8 ppm indicated that the compound had  , -O-H, -C-O-CH_3, F-CH_3,  and  respectively (Figure 4). Peak values of ^{13}C NMR spectrum of the same purified compound indicated the presence of ether (55-90 ppm), alcohol (45-90 ppm), amines (10-70 ppm) and aromatic compound (90-160 ppm) (Figure 5). The molecular formula of the compound has been documented as $C_{28}H_{26}N_4O_3$ on the basis of elemental analysis. On the basis of spectral and other supportive data, the antimicrobial compound of the *S. champavatii* (KV2) was identified as "*Staurosporine*" (Fig. 6).

Antimicrobial activity of pure compounds of Staurosporine: The maximum antibacterial inhibitory effect of staurosporine compound produced by *S. champavatii* (KV2) was exhibited on *E. coli* (18 mm), followed by *B. subtilis* (17 mm), *K. pneumoniae* (16 mm), *S. aureus* (14 mm), *S. pneumoniae* (12 mm) and *S. typhi* (12 mm). The maximum antifungal inhibitory effect of staurosporine was exhibited on *Fusarium* sp. (17 mm), followed by *A. fumigatus* (14 mm), *A. niger* (12 mm) and *A. flavus* (11 mm.

Table 1. Preliminary and secondary screening of antimicrobial activity of actinobacterial isolates

S. No.	Isolate code	Primary screening									
		Diameter of inhibition zones (mm)									
		B. subtilis	*S. aureus*	*S. pneumoniae*	*E. coli*	*K. pneumoniae*	*S. typhi*	*Fusarium* sp.	*A. niger*	*A. flavus*	*A. fumigatus*
1.	KV1	4	6	7	5	4	6	8	12	11	3
2.	KV2	12	7	18	5	9	18	13	9	7	16
3.	KV3	-	-	-	-	-	5	-	-	9	12
4.	KV4	19	2	5	12	20	4	5	6	-	10
Secondary screening											
1.	KV1	10	10	-	13	-	-	-	7	10	3
2.	**KV2**	**13**	**14**	**7**	**20**	**16**	**15**	**8**	**11**	**14**	**7**
3.	KV4	5	-	-	8	10	5	-	-	-	-

Table 2. Antimicrobial efficacies of *S. champavatii* (KV2)

S. No.	Name of the solvent	Diameter of inhibition zones (mm)									
		B. subtilis	*S. aureus*	*S. pneumoniae*	*E. coli*	*K. pneumoniae*	*S. typhi*	*Fusarium* sp.	*A. niger*	*A. flavus*	*A. fumigatus*
1	Acetone	9	10	-	15	9.5	-	9	11	7	9
2	Chloroform	10	12	8	-	15	12	-	8	10	8
3	Ethanol	7.5	6.5	8.5	10	11	12	-	-	9	10
4	Ethyl acetate	9	6	12	14	-	8.5	10	9.5	9	13
5	Methanol	7.5	9.5	9	8.5	12	8.5	6.5	-	7	9
6	Petroleum ether	10	11.5	11	6.5	9.5	10	-	9	6	10
7	Xylene	11.5	7.5	13	9	11	11	-	-	10	11

Table 3. Cultural characteristics of selected actinobacteria on different culture media

S. No.	Name of the medium	*S. champavatii* (KV2)
1	**Starch casein agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Light Grey Light Brown Nil
2	**Oat meal agar** (ISP 3)	
	Aerial mycelium Substrate mycelium Pigmentation	Dull white White Nil
3	**Kenknight agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Light Grey Greyish Brown Nil
4	**Actinomycetes isolation agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Grey Light white Nil
5	**Peptone yeast agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Grey Pale yellow Nil
6	**Glucose yeast extract peptone agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Grey Light Yellow Nil
7	**Nutrient agar**	
	Aerial mycelium Substrate mycelium Pigmentation	Dull white Light yellow Nil

Table 4. Extractability of antimicrobial compounds produced by *S. champavatii* (KV2)

S.No.	Name of the solvent(s)	Staurosporine [*S. champavatii* (KV2)]		S.No.	Name of the solvent(s)	Staurosporine [*S. champavatii* (KV2)]	
		Colour of the extract	Colour intensity			Colour of the extract	Colour intensity
1.	Acetone	Colourless	-	5.	Methanol	Pale yellow	+
2.	Chloroform	Pale yellow	+	6.	Petroleum ether	Colourless	-
3.	DMSO	Brown colour	+++	7.	Xylene	Colourless	-
4.	Ethyl acetate	Light brown	++	8	Distilled water	Colourless	-

- = No dissolving; + = minimum dissolving; ++ = moderate dissolving; +++ = maximum dissolving

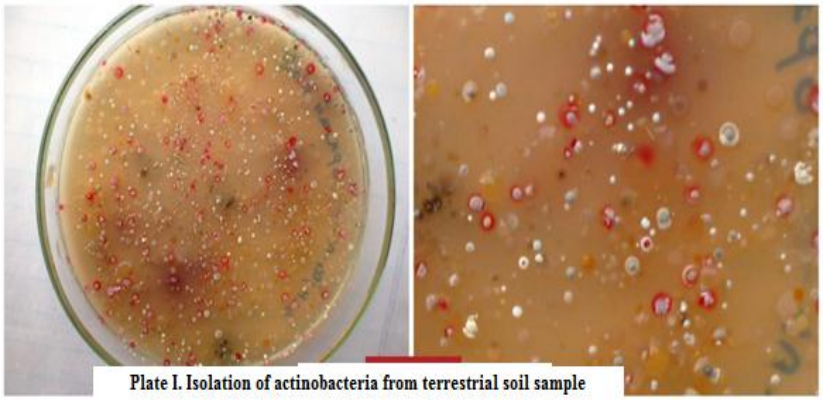


Plate I. Isolation of actinobacteria from terrestrial soil sample

Plate II. Colony morphology of actinobacterial isolate KV2 fro terrestrial soil

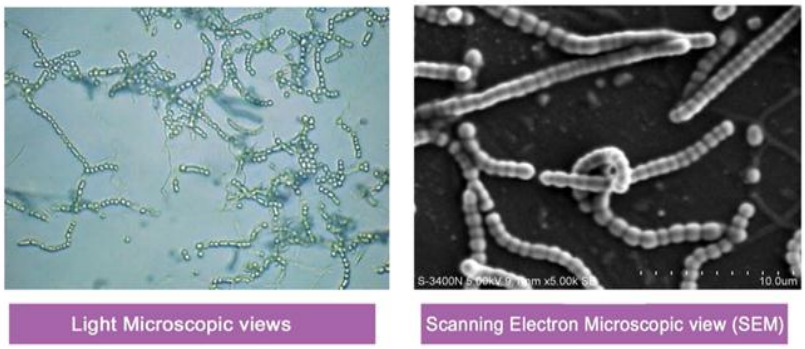


Plate III. Microscopic views of Streptomyces sp. (KV2)

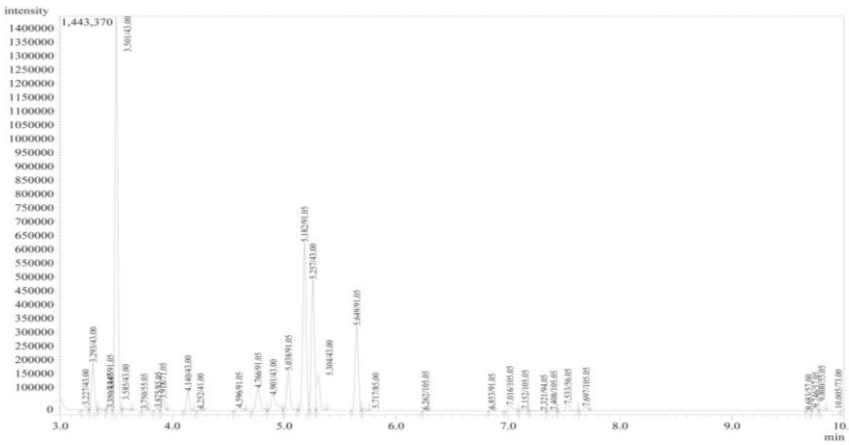


Figure 1. GC-MS analysis of compounds produced by *Streptomyces* sp. (KV2)

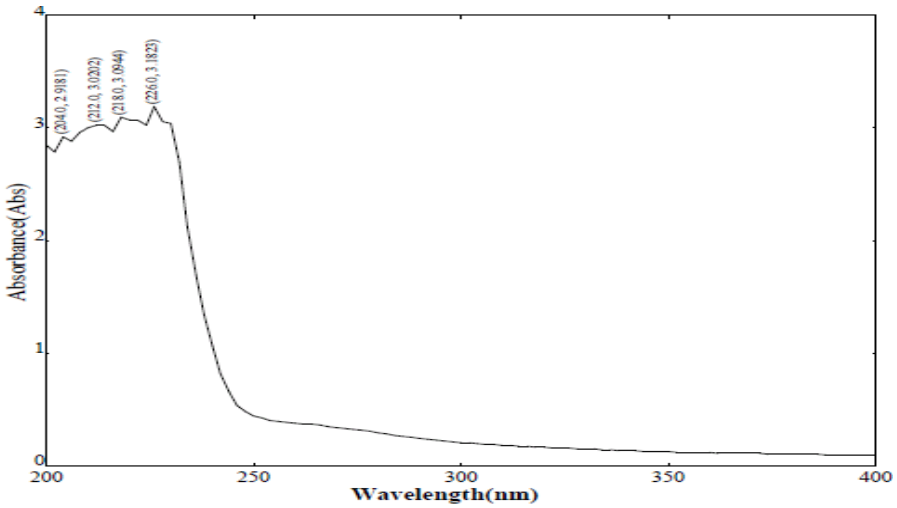


Figure 2. UV spectrum analysis of the *Streptomyces* sp. KV2 compound.

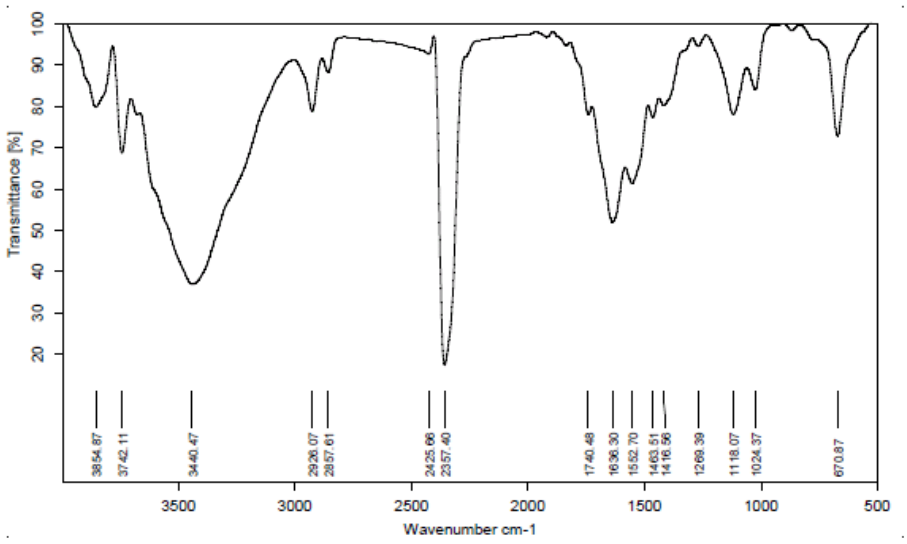


Figure 3. FTIR spectrum analysis of the *Streptomyces* sp. KV2 compound.

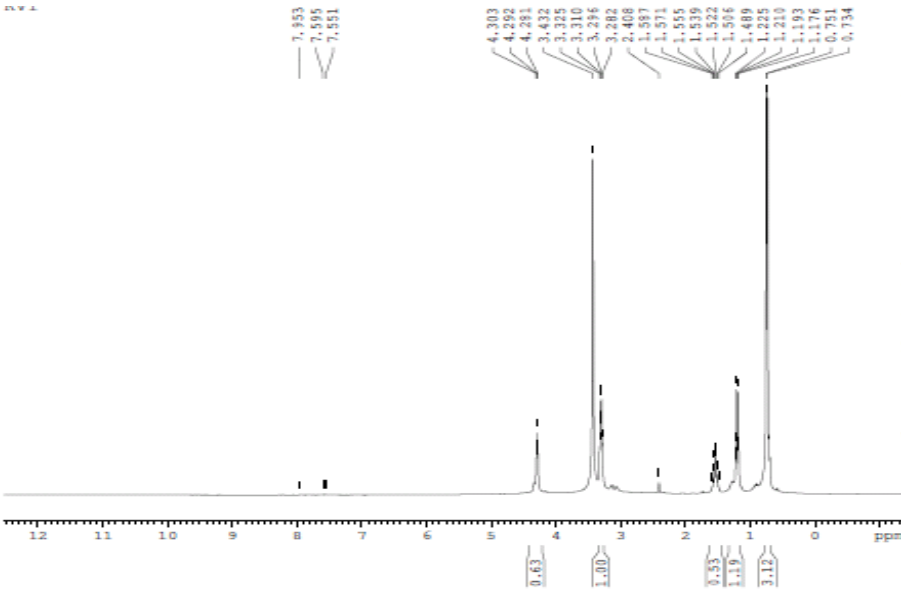


Figure 4. ^{1}H NMR analysis of *Streptomyces champavatii* (KV2)

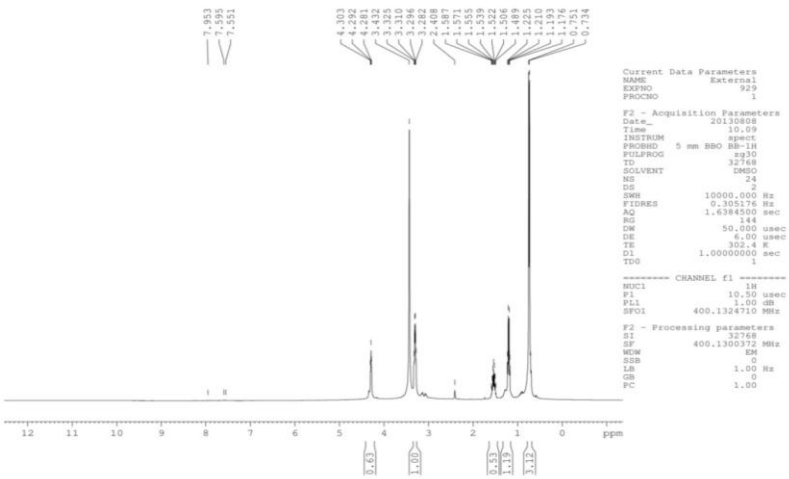


Figure 5. ^{13}C NMR analysis ofantimicrobial compound of *Streptomyces champavatii (*KV2)

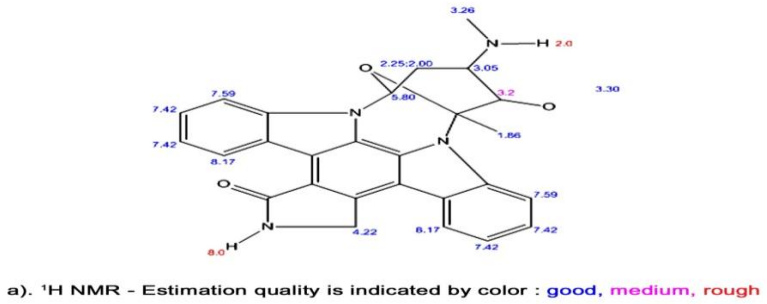


Figure 6. Structure of staurosporine by *Streptomyces champavatii (*KV2)

DISCUSSION

Actinobacteria are the dominant group of soil population together with bacteria and fungi and are originally considered as an intermediate group between bacteria and fungi. They are free living saprophytic bacteria, and a major source for production of antibiotics. They also play a major role in recycling of organic matter (Unaoguet *et al.*, 1994), production of novel pharmaceuticals, nutritional materials, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. Around 80% of the total antibiotic production has been obtained from *Streptomuces* (Wellington *et al.*, 1992). During the last few decades, actinobacteria have become the most creditable source for antibiotics. In the 20th century, 75-80% of the entire discovered antibiotics were derived from the order actinomycetales, mainly from *Streptomyces* species (Thakur *et al.*, 2007). Furthermore, some 10-20 compounds are used in agriculture mainly as pesticide, herbicides, plant protecting agents and food additives (Moncheva *et al*., 2002).

Actinobacteria play an important role in soil bio-geochemical processes which determine plant productivity, successful functioning of introduced microbial bioinoculants and their influence on soil health. Exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behavior in soil habitats (Hill, 2000). The wide distribution of *Streptomyces* in soil and their

ability to produce novel antibiotics and non-antibiotic lead molecules had caused these bacteria to be targeted in drug screening programme. Discovery of novel antibiotics from actinobacteria are important in helping to cope with the growing proportion of antibiotic-resistant bacterial infections that become untreatable. Further, the frequency of novel bioactive compounds discovered from terrestrial actinobacteria decreases with recent time, because much attention has been focused on screening of actinobacteria from diverse environments for their ability to produce new secondary metabolites (Ravikumar *et al.*, 2011). Thus, the present study has been designed to isolate the terrestrial actinobacteria, screened their antimicrobial potentials against some bacterial and fungal organisms, and identified the active principles of the antimicrobial compounds. So that, the study has proved that, the terrestrial soil is an authentic source of novel antibiotics.

The isolation and characterization of microbial species are as important to understanding their existence in natural ecosystems. The isolation of diverse and novel actinobacteria provides a theoretical guide for the exploitation and utilization of actinobacterial resources (Li *et al*., 1996). Initially, actinobacteria were characterized on the basis of morphological characters so as to have a preliminary determination of the genus. Actinobacteria can be observed under the light microscope using coverslip culture (Vijayakumar *et al.*, 2007; Khan, 2008; Arifuzzaman *et al.*, 2010) and slide culture techniques (Kavitha and Vijayalakshmi, 2007). They are observed for several characters such as presence or absence of aerial and substrate mycelia, fragmentation or non fragmentation of substrate and aerial mycelium, presence of sclerotia, spore chain morphology and color of aerial spore mass (Kavitha and Vijayalakshmi, 2011).

In the present study, 50 different isolates were selected based on their morphological characteristics (colonial and microscopic). Colour of the aerial spore mass of the isolates were categorized into 2 groups including white and brown series on SCA medium. Similar result had been reported by Vijayakumar *et al.* (2007; 2008). The colonies appeared with a leathery or chalky texture, folded appearance and were branching, filamentous with/without aerial mycelia (Oskay *et al*., 2004). Hence, the colours of aerial spore mass and reverse sides of the actinobacteria grown on culture media could vary depending on the nutrients provided.

Based on the colonial and microscopic morphology, predominant isolates of the present study (66%) belonged to the genus *Streptomyces.* It has been reported by many workers (Vijayakumar, 2006; Vijayakumar *et al.*, 2007; 2009; Thirumurugan and Vijayakumar, 2012; 2013a; Cholarajan and Vijayakumar, 2012; 2013a) that, the streptomycetes are the predominant soil actinobacteria in both terrestrial and marine environments.

Characterization of actinobacteria

If an organism is considered for the welfare of the human beings and agriculture or industrially significance, it becomes imperative that the taxonomic confirmation of the organisms is much more essential. Identification of microorganisms, especially streptomycetes, could be confirmed by morphological, cultural, biochemical, antigenic, metabolic, genetic and molecular characteristics as per ISP description.

In the present study, two actinobacterial isolates namely *Streptomyces* sp. (KV2) were found to produce rectiflexibiles sporophore and spore surface was smooth. Similar type of result has been reported by Moncheva *et al*. (2002). It was also supported by Atalan *et al.* (2000) that, the smooth surface spores are characteristics of 75 to 80% of streptomycetes.

The chemical composition of medium has a bearing on the morphology of organisms. The actinobacterial isolates were grown on various culture media *viz*., SCA, oat meal agar, kenknight agar, actinomycetes isolation agar, peptone yeast agar, glucose yeast extract peptone and nutrient agar medium. Among them, SCA was better than other media tested, because it was found that actinobacterial isolates could utilize all nutrients and sporulate in mass. Similar type of result has been reported by Moncheva *et al*. (2002).

Various biochemical characteristics of the streptomycetes are used for identification (Kim and Goodfellow 2002; Manfio *et al.*, 2003). In the present investigation, various biochemical tests were carried out to differentiate two potential antimicrobial compound producing actinobacteria.

Comparatively, Slack *et al.* (1969) characterized as many as 64 strains of *Actinomyces israelii* by using biochemical properties and categorized into several subgroups. None of the strains was positive for catalase, indole production and in the Voges-Proskauer test; 90% were methyl red-positive and 62% were nitrate-positive. Acid was produced by all the isolates from glucose and xylose (each 100%), salicin (98%), raffinose (95%), lactose (89%), cellobiose (83%), mannose (78%), arabinose (76%), inositol (58%), mannitol (48%) and starch (31%), *A. israelii* can be identified by the fluorescent-antibody method, but there was no single morphological or biochemical characteristics which could be used for its identification. Then, most of the isolates were efficient in hydrolysing starch and casein (Ravel *et al*., 2000) except a few strains. Based on the present and previous studies, it is concluded that, the biochemical properties of the actinobacteria varied depending on the nutrients supplied in biochemical media and biochemistry of an organism; hence this aspects could potentially be used as taxonomic criterion for genus level identification.

In the present study, the two potential actinobacterial isolates namely *Streptomyces* sp. (KV2) was selected for molecular characterization. 16S rRNA gene sequence analysis of the two isolates showed major variations and phylogenetic tree with bootstrap value of the sequences of the isolates showed distinct phylogenetic position within the representatives of the stretomycetes. *Streptomyces* sp. (KV2) found 98% similarity with the existing species of *S. champavatii* iafA.

Based on the morphological, biochemical, cultural and molecular characterization, the potential isolate *Streptomyces* sp. (KV2) isolated from Kanniyakurichy (Thanjaur District) is closely related to the existing species of *S. Champavatii*.

It is also evident that 16S rRNA gene sequencing has played a vital role in the identification of actinobacteria by many workers (Kim *et al.,* 2004; Song, 2004; Dhanasekaran *et al.,* 2005a; Vijayakumar, 2006; Vijayakumar *et al.,* 2012a; b; Meena *et al.,* 2013). In the present study, distinct variation in the secondary structure, G+C composition, presence of restriction enzymes sites in 16S rRNA gene sequence of two different *Streptomyces* isolates showed molecular level specificity of each and every individual isolates. For the development of universal identification system of not only actinobacteria, but all microorganisms, a polyphasic taxonomic approach utilizing morphological, biochemical, cultural, ecological and molecular characteristics will help taxonomists for the development of meaningful taxonomic identification system.

The present investigation revealed that *S. champavathi* (KV2) could produce maximum antimicrobial compounds in starch casein broth. The separation of antimicrobial compounds of *Streptomyces* spp. was carried out by using chromatographic techniques. Similar appraoch have been attempted by many workers (Mathivanan and Murugesan, 1999; Augustine *et al.,* 2005; Vijayakumar *et al.,* 2012a; b). It has been highlighted that the discovery of an antibiotic could be attained by screening of microorganisms, solvent selection and extraction, separation and purification.

Characterization of antimicrobial compound

The functional groups of antimicrobial compounds were evaluated by various qualitative biochemical tests. The present study revealed the presence of aldehyde and ketone, protein, peptide and amino acid, and absence of sugar moiety, reducing sugar and pentose sugar in *S. champavathi* (KV2). Hence, it is evident that the compound had alcohols, phenols and alkenes group which was supported with the results of Harindran *et al.* (1999) and Datta *et al.* (2001).

GC-MS analysis identified the following major compounds from *S. champavathi* (KV2) such as acetic acid, 1-methylpropyl ester (CAS) 2-butyl acetate, sec-butyl acetate sec. -butylacetat, acetic acid, 2-methylpropyl ester (CAS) isobutyl acetate, benzene, ethyl- (CAS) EB ethylbenzene, methyllaurate, benzene, 1,2-dimethyl- (CAS) o-xylene, benzene, 1,4-dimethyl- (CAS) p-xylene, benzene and 1,3-dimethyl- (CAS) m-xylene etc. Similar results have been reported by Charlotte *et al.* (2002) and Rajesh *et al.* (2013).

The pale yellowish antimicrobial compound of *S. champavatii* (KV2) was viscous in nature, and its melting point was 145°C. It was stable at pH 4-7 and temperature 30-45°C. The compound was stable at pH 5-7 and temperature 25-45°C. Ultimately, based on the UV, FT-IR and ^{13}C and ^{1}H spectral studies, the antimicrobial compounds of *S. champavatii* (KV2) were identified as *Staurosporine*. The molecular formulas and molecular weights of the antimicrobial compound staurosporine are $C_{28}H_{26}N_4O_3$ and 466.53 kDa.

In accordance to this study, the bioactive compounds namely, cyclic depsipeptide valinomycin, indolocarbazole alkaloid staurosporine and butenolide with anti-parasitic and anti-infective activities has already been reported from marine *Streptomyces* sp. of Mediterranean sponges (Elardo *et al.*, 2012). In the present study, staurosporine were dereived from streptomycetes of terrestrial origin. Hence, it is suggested that re-evaluation of previously known compounds for novel anti-infective activities could be achieved by the routine isolation and screening of actinobacteria from diverse habitats. Similar results have been reported from the terrestrial actinobacteria by many workers (Swaadoun *et al.*, 1999; Ilic *et al.,* 2005; Mohamed Shaaban *et al.,* 2008).

Antimicrobial assay of antimicrobial compound derived from *Streptomyces* spp.

The purified antimicrobial compounds derived from *S. champavatii* (KV2) namely staurosporine were tested against pathogenic bacteria and fungi. Thus, the antimicrobial compounds of both the isolates exhibited maximum antibacterial activity than antifungal activity. Similarly, Vijayakumar (2006) and Vijayakumar *et al.* (2012a) have also been reported that, the pure antimicrobial compounds derived from marine streptomycetes found maximum activity against bacteria. In contrast to this, Dhanasekaran (2005) reported maximum antifungal activity than antibacterial activity of antmicrobial compounds produced by marine streptomycetes.

Generally, bioavailability of chemicals, e.g. heavy metals or pesticides, is an important issue of soil health because of its impact on microbial activities. Indicators of soil health have further been defined as measurable proxy for environmental processes that collectively convey whether the soil is functioning normally. Thus, the present investigation clearly revealed the biodiversity of actinobacteria in terrestrial soil and their antimicrobial potentials against human and some soil borne fungi. Further, to implement these findings for human welfare,

it is necessary to carryout clinical trials and strategies for optimization of large scale production of both cell biomass and antimicrobial compounds. Ultimately, it is recommended that, the streptomycetes could be acted as soil antagonistic organisms for the health of the soil. It is also used to control the soil borne phyto-pathogens, and protection of soil from contaminations. Further, they also could be acted as a potential source of novel antibiotics against human pathogenic microorganisms with great pharmaceutical value.

Therefore, the present investigation offers an idea about the diversity of antimicrobial compound producing actinobacteria in the terrestrial soil ecosystem of Thanjavur district, Tamilnadu. They are distinguished by using different phenotypic and genotypic characteristics. The separation, purification and identification of antimicrobial compounds and the chemistry of the compounds from *S. champavatii* (KV2) are established.

The finding of present study emphasizes the role of terrestrial *Streptomyces* spp. in the production of novel antimicrobial compounds. Optimization of media and other physico-chemical parameters will lead to derivation of novel antimicrobials from diverse actinobacteria. The findings in our study are an experimental authentication of this concept.

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